THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 23, pp. 15857–15866, June 5, 2009

Wnt-5a/JNK Signaling Promotes the Clustering of PSD-95 in Hippocampal Neurons*[S]

Received for publication, December 1, 2008, and in revised form, March 6, 2009 Published, JBC Papers in Press, March 30, 2009, DOI 10.1074/jbc.M808986200

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During the formation of synapses, specific regions of pre- and postsynaptic cells associate to form a single functional transmission unit. In this process, synaptogenic factors are necessary to modulate pre- and postsynaptic differentiation. In mammals, different *Wnt* ligands operate through canonical and non-canonical *Wnt* pathways, and their precise functions to coordinate synapse structure and function in the mature central nervous system are still largely unknown. Here, we studied the effect of different *Wnt* ligands on postsynaptic organization. We found that *Wnt-5a* induces short term changes in the clustering of PSD-95, without affecting its total levels. *Wnt-5a* promotes the recruitment of PSD-95 from a diffuse dendritic cytoplasmic pool to form new PSD-95 clusters in dendritic spines. Moreover, *Wnt-5a* acting as a non-canonical ligand regulates PSD-95 distribution through a JNK-dependent signaling pathway, as demonstrated by using the TAT-TI-JIP peptide in mature hippocampal neurons. Finally, using adult rat hippocampal slices, we found that *Wnt-5a* modulates glutamatergic synaptic transmission through a postsynaptic mechanism. Our studies indicate that the *Wnt-5a*/JNK pathway modulates the postsynaptic region of mammalian synapse directing the clustering and distribution of the physiologically relevant scaffold protein, PSD-95.

During the formation of synapses, pre- and postsynaptic sides contain specific molecules that are involved in the regulation and plasticity of synaptic transmission (1–3). Although much is known about the molecular mechanisms of synaptic differentiation, major gaps remain in our understanding of the process, particularly with regard to the signals mediating the structuring of the postsynaptic apparatus of central mammalian synapses (2, 4). At excitatory synapses, the postsynaptic side is characterized by an electrodense thick matrix, called postsynaptic density (PSD).[3] The PSD contains key molecules involved in the regulation of glutamate receptor targeting and trafficking (1, 5). There is considerable interest in elucidating the molecular mechanism that controls synaptic targeting and trafficking of these proteins in the postsynaptic region because of their essential role in synaptic plasticity (6). Moreover, in neurodegenerative pathologies, such as Alzheimer disease, it has been evidenced that the postsynaptic region, including several proteins of the PSD, is the primary target of the synaptotoxic effect of the amyloid β-peptide (7–9).

Wnt signaling is essential for neuronal development and the maintenance of the nervous system (10–12). *Wnt* regulates synapse formation; in fact, the pioneering work of Salinas and co-workers (12–15) established that *Wnt-7a* induces the clustering of presynaptic proteins in young primary cerebellar cultures. Also, *Wnt* ligands regulate neurogenesis of hippocampal stem cells in the adult rat (16), and *Wnt-3a* modulates long term potentiation in mouse hippocampal slices (17, 18).

The expression of *Wnt* ligands and proteins of the *Wnt* signaling machinery in the mature nervous system (19) suggests that *Wnt* signaling plays a key role in neuroprotection and synaptic plasticity, in addition to its role in neurite patterning in the developing nervous system (20–22). Indeed, *Wnt* ligands can act focally to regulate changes in neuronal cell shape and synaptic terminals, which are thought to underlie changes in synaptic function and learning; thus, *Wnt* ligands would appear to be particularly well suited as mediators of synaptic plasticity.

Recently, we evaluated the role of *Wnt* ligands in the presynaptic differentiation and function, and we found a short term modulation of the synaptic vesicle cycle and synaptic transmission in mature hippocampal neurons by *Wnt-7a* and *Wnt-3a* but not by *Wnt-5a* (23). However, whether *Wnt* ligands are able to modulate the postsynaptic region is still unknown.

Wnt proteins signal through at least three different pathways. The binding of *Wnt* proteins to Frizzled (Fz) receptors results in the activation of the scaffold protein Dishevelled (Dvl). In the canonical pathway, *Wnt* proteins signal through Dvl to increase cytoplasmic β-catenin levels, and then β-catenin enters the

* This work was supported by Fondo de Financiamiento de Centros de Excelencia en Investigación, Comisión Nacional de Investigación Científica y Tecnológica, and the Millennium Institute (to N. C. I.); Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) Grant 1061074 and DIPUV Grant CID/1/2006 (to C. B.); and predoctoral fellowships from FONDECYT (to G. G. F., I. E. A., W. C., and C. P. G.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

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[3] The abbreviations used are: PSD, postsynaptic density; DIV, day(s) *in vitro*; HEK-293, human embryonic kidney 293; JNK, c-Jun NH_2-terminal kinase; ACSF, artificial cerebrospinal fluid; fEPSP, field excitatory postsynaptic potential; PPF, paired pulse facilitation; BSD-X, bisindolylmaleimide-X; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HA, hemagglutinin.

nucleus, where it co-activates transcription of *Wnt* target genes (24, 25). Non-canonical *Wnt* signaling pathways have been described that do not affect gene transcription through β-catenin but instead mediate several cellular processes through different molecular intermediates, including monomeric GTPases of the Rho family, mitogen-activated protein kinases like Jun N-terminal kinase (JNK), and changes in intracellular calcium levels (26).

In the present study, we tested whether different *Wnt* ligands are able to modulate the assembly and differentiation of the postsynaptic regions in mature hippocampal neurons. We report here that *Wnt-5a*, but not *Wnt-7a*, regulates postsynaptic structure and protein localization, directing the clustering of PSD-95 (postsynaptic density protein-95) through the *Wnt*/JNK pathway. Moreover, we show that *Wnt-5a* modulates the synaptic transmission. These results suggest that the noncanonical *Wnt*/JNK pathway controls synaptic structure and function of the excitatory postsynaptic compartment of mammalian synapse.

EXPERIMENTAL PROCEDURES

Reagents—Formylated hexapeptide was obtained from Genemed Synthesis, Inc. (South San Francisco, CA); fumagillin, anisomycin, palmitate, and 2-bromopalmitate were from Sigma; bisindolylmaleimide-X (BSD-X), SP600125, KN-93, and TAT-TI-JIP-(153–163) were from Calbiochem; rabbit anti-synapsin-1, mouse anti-β-catenin, mouse anti-transferrin, rabbit anti-β-tubulin, rabbit anti-GAPDH, mouse anti-N-cadherin, mouse anti-CAMKII, goat anti-PSD-95, and goat anti-*Wnt-5a* were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); mouse anti-PSD-95 was from the University of California/NINDS/NIMH, National Institutes of Health (Davis, CA); rabbit anti-JNK and rabbit anti-phospho-JNK were from Cell Signaling Technology (Beverly, MA); antibodies and secondary antibodies labeled with ^{488}Alexa, ^{543}Alexa, or ^{633}Alexa were from Affinity Bio Reagents Inc. (Golden, CO); and phalloidin labeled with ^{633}Alexa was from Molecular Probes, Inc. (Eugene, OR).

Constructs—Green fluorescent protein (GFP) was from Clontech (Mountain View, CA); the different hemagglutinin-*Wnt* constructs were a kind gift of several individuals that made this work possible. HA-*Wnt-7a* construct was a gift from Dr. Patricia Salinas (University College London, London, UK); HA-*Wnt-5a* was a gift of Dr. Randall Moon (University of Washington, Seattle, WA); and sFRP-1 (soluble Frizzled receptor protein-1) was a gift of Dr. Jeremy Nathans (The Johns Hopkins University School of Medicine, Baltimore, MD).

Conditioned Medium Containing Wnt Ligands—To generate secreting *Wnt* ligands, Human embryonic kidney 293 (HEK-293) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen), 100 μg/ml streptomycin, and 100 units/ml penicillin. HEK-293 cells were transiently transfected by calcium phosphate precipitation with constant and equal amounts of empty vector pcDNA (control) or pcDNA containing sequences encoding *Wnt-7a*, and *Wnt-5a* and sFRP-1-HA constructs (27) or stably transfected HEK-293 cells containing pcDNA or *Wnt-5a* ligands were used. Then transfected HEK-293 cells were maintained in neurobasal medium supplemented with 100 μg/ml streptomycin and 100 units/ml penicillin for 3 days. The presence of *Wnt* ligands in the conditioned medium was verified by Western blot analysis using an antibody against the HA epitope. To block *Wnt* ligand function, we co-incubated the different *Wnt* ligands with sFRP-1 for 30 min before treatment of the neurons.

Hippocampal Neuronal Cultures and Transfection—Rat primary hippocampal neurons were obtained from 18-day-old Sprague-Dawley rat embryos (as described previously) (27) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% horse serum for 2 h. The culture medium was then substituted with Neurobasal medium supplemented with B27, 100 μg/ml streptomycin, and 100 units/ml penicillin. Cells were treated with 2 μM 1-β-D-arabinofuranosylcytosine for 24 h on day 3 to reduce the number of proliferating non-neuronal cells. Experiments were performed at 14–18 DIV in the presence or absence of pcDNA, *Wnt-5a*, *Wnt-7a*, or sFRP conditioned medium, preincubation with or without fumagillin (10–100 nM) for 16 h, KN-93 (10 μM), BSD-X (1 μM), SP600125 (0.5 μM), anisomycin (1 μg/ml), and TAT-TI-JIP (0.1–1 μM) co-incubated or not with *Wnt-5a* conditioned medium for 1 h. Hippocampal neurons expressing GFP were transfected with enhanced GFP at 3 DIV with Lipofectamine and treated with the conditioned media at 14 DIV or fumagillin.

Immunofluorescence and Image Analysis—Hippocampal neurons were subjected to different treatments while on coverslips within 24-well plates at a plating density of 30,000 cells/coverslip, fixed with 4% paraformaldehyde, 4% sucrose in PBS for 20 min, permeabilized with 0.2% Triton X-100 for 5 min, blocked with 0.2% gelatin, and stained with PSD-95 antibody. Phalloidin coupled to Alexa 633 was used as a neurite marker. Digital images of neurons on coverslips were captured with a Zeiss confocal microscope using a ×63/1.4 numerical aperture oil immersion objective. Images used for quantification were taken with identical microscope settings and analyzed using Image J software (National Institutes of Health, Baltimore, MD). PSD-95 images from 10 microscope fields for each condition of three independent experiments were registered. Each field containing processes for 1–2 neurons, in which 2–3 neurites/neurons staining with phalloidin to label neuronal processes, were selected. To quantify PSD-95 cluster parameters, images of individual neurites were isolated, background for neurite-free fields was subtracted, and the mean intensity of diffuse dendritic PSD-95 staining was obtained using multiple regions of interest from dendritic shafts (28). In each image, punctate PSD-95 staining was segmented using a value of 3 times the local mean intensity of diffuse shaft staining as a lower limit threshold. PSD-95 cluster number, mean intensity, integrated intensity, and size were obtained with the particle analysis tool using size particle limits of 0.04–1 μm^2. Cluster number was normalized against neurite length to obtain cluster density. Integrated intensities of PSD-95 clusters were added and normalized against total dendritic PSD-95 integrated intensity to obtain relative cluster integrated intensity. For quantification of dendritic spine density, images of neurites of transfected neurons were captured with a 2× digital zoom. Dendritic protrusions below 3 μm in length showing visible

spine heads and positives for PSD-95 immunoreactivity were quantified using the Image J software (National Institutes of Health) from at least 30 neurites for each condition in three independent experiments.

Subcellular Cell Fractionation—Hippocampal neurons were treated with *Wnt-5a* ligand or pcDNA for 2 h or with palmitate (10 μM) for 8 h or 2-bromopalmitate (10 μM) for 4 h. Then total protein was lysed in a buffer containing 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and 1 mM dithiothreitol, supplemented with protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin). Lysates were incubated in ice for 30 min and centrifuged at 700 $\times$ *g* for 5 min at 4 °C. Supernatants were centrifuged at 100,000 $\times$ *g* for 1 h at 4 °C, and the pellet (equivalent to membrane or particulate fraction) was resuspended in a buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% Triton X-100, 0.15 mM NaCl, and the above protease inhibitor mixture (27). The supernatant (equivalent to the cytoplasmic fraction) and the enriched membrane fraction were resolved using SDS-PAGE, the proteins were transferred to polyvinylidene difluoride membranes, and immunoblotting was done using monoclonal anti-PSD-95. Controls of fractionation were performed using anti-GAPDH and β-tubulin antibodies (used as markers of soluble proteins) and anti-transferrin receptor and anti-N-cadherin antibodies (used as markers of membrane-associated proteins).

Slice Preparation and Electrophysiology—Hippocampal slices were prepared according to standard procedures from 22–30-day-old Sprague-Dawley rats. Transverse slices (250–300 μm) from the dorsal hippocampus were cut under cold artificial cerebrospinal fluid (ACSF) using a Vibroslice microtome (VSL, WPI) and incubated in ACSF for more than 1 h at room temperature. In all experiments, picrotoxin (10 μM) was added to ACSF perfusion media in order to suppress inhibitory $GABA_A$ transmission. Then slices were transferred to an experimental chamber (2 ml), superfused (3 ml/min at 22–26 °C) with gassed ACSF, and visualized by transillumination with a binocular stereo microscope (MSZ-10; Nikon). The experiments were carried out at room temperature (21–22 °C), measured at the recording chamber. Extracellular field potential recordings (23) were made with glass pipettes, connected to an AC amplifier (P-5 Series; Grass), with gain $\times$10,000, LP filter (3.0 kHz), and HP filter (0.30 Hz) that was placed in the middle of the stratum radiatum of CA1. The electric pulses (50 μs, 0.3 Hz, 20–100 μA) were applied on Schaeffer collaterals, eliciting compound action potentials from the presynaptic axons (fiber volley) followed by field excitatory postsynaptic potentials (fEPSPs). To evoke fEPSPs, Schaeffer collateral fibers were activated by bipolar cathodic stimulation, generated by a stimulator (Master 8; AMPI) connected to an isolation unit (Isoflex; AMPI). The bipolar concentric electrodes (platinum/iridium, 125 μm OD diameter; FHC Inc.) were placed in the stratum radiatum within 100–200 μm of the recording site.

The paired pulse facilitation (PPF) index was calculated by $(R2 - R1)/R1$), where *R1* and *R2* are the peak amplitudes of the first and second fEPSPs, respectively. Evoked postsynaptic responses were analyzed off-line, using analysis software (Minianalysis; Synaptosoft), which allowed visual detection of events, computing only those events that exceeded an arbitrary threshold. Data are expressed as means $\pm$ S.E. (number of slices), unless otherwise noted. Student's *t* test analysis at $p < 0.05$ determined significant differences between control and experimental treated groups.

TABLE 1

***Wnt-5a*, but not *Wnt-7a*, affects specifically the postsynaptic protein PSD-95 without affecting the presynaptic protein Synaptophysin**

Quantification of PSD-95 and synaptophysin clusters in 100 μm of neurite length of hippocampal neurons 14 DIV exposed to different treatments ($n = 3$).

Treatment	PSD-95 clustering	Synaptophysin clustering
	number/100 μm neurite	*number/100 μm neurite*
pcDNA	59.67 $\pm$ 7.72	45.32 $\pm$ 8.93
Wnt-7a	64.17 $\pm$ 10.03	86.07 $\pm$ 16.35[a]
Wnt-5a	110.72 $\pm$ 19.01[a]	48.79 $\pm$ 12.16

[a] $p < 0.01$.

Statistical Analysis—Data were expressed as the mean $\pm$ S.E. of the values from the number of experiments, as indicated in the corresponding figures. Data were evaluated statistically by using Student's *t* test, with $p < 0.01$ or $p < 0.05$ considered significant.

RESULTS

Wnt-5a Affects Clustering of PSD-95—Several *Wnt* ligands, including *Wnt-4, Wnt-5a, Wnt-7a*, and *Wnt-11*, are expressed in the hippocampus of adult rats and in hippocampal neuron cultures using a prototype mammalian *Wnt* gene for screening (23). Previously, we have shown that short term exposure to *Wnt-7a* but not *Wnt-5a* ligand modulates the synaptic vesicle cycle and synaptic transmission in hippocampal neurons (23). Here, we examined whether *Wnt* ligands can regulate the postsynaptic region in mature hippocampal neurons. Neurons exposed to *Wnt-7a* ligand for 1 h did not show any changes in the clustering of the postsynaptic protein PSD-95, with respect to the control (Table 1) under conditions in which the clustering of the presynaptic protein synaptophysin was increased (Table 1), as described previously (23). However, when hippocampal neurons were incubated with the *Wnt-5a* ligand, an increase in the PSD-95 density was observed (Fig. 1*A*, *a*, *b*, and *d*, and Table 1) without affecting the clustering of the presynaptic protein synaptophysin (Table 1).

To establish the specificity of the *Wnt-5a* ligand conditioned medium effect, *Wnt-5a* ligands were incubated with a soluble Fz receptor protein (sFRP) antagonist. sFRP binds to the *Wnt* ligands, thereby preventing their interaction with cellular membrane-bound receptors (29). The co-treatment of *Wnt-5a*/sFRP prevented the increase of the number of PSD-95 clusters triggered by *Wnt-5a* (Fig. 1*A*, *b*, *c*, and *d*). Moreover, to ensure that the effect in PSD-95 clustering is due to *Wnt-5a* and not to the presence of other proteins contained in the conditioned medium, we used a formylated hexapeptide ligand derived from the sequence of the *Wnt-5a* ligand that mimics the full *Wnt-5a* molecule in other systems (30). We found a similar increase in the number of PSD-95 clusters when hippocampal neurons were exposed to 50 μM formylated hexapeptide ligand for 1 h of treatment under conditions in which the hexapeptide ligand did not affect the clustering of the presynaptic protein synapsin-1 (Fig. S1).

The short term effect of the *Wnt-5a* ligand in the clustering of PSD-95 was evaluated; a time-dependent increase in PSD-95

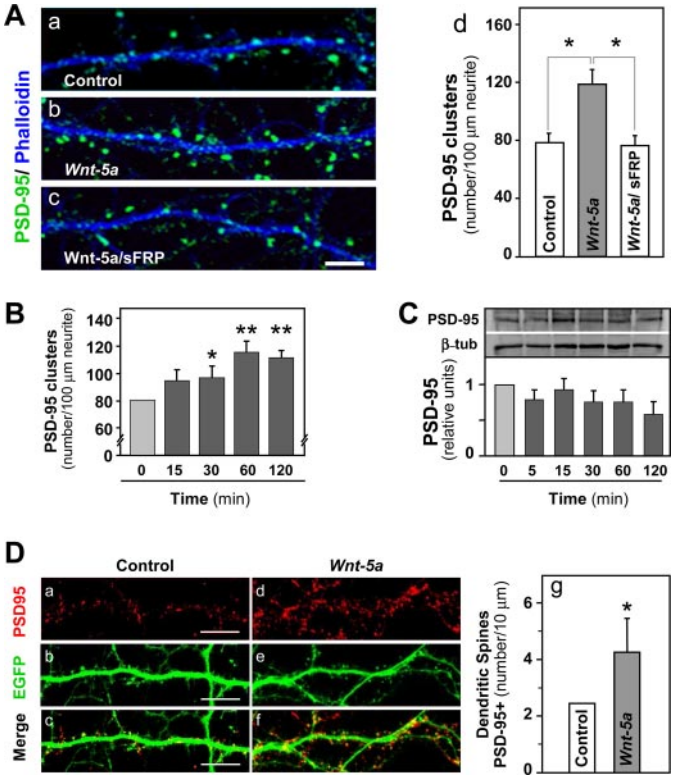

FIGURE 1. ***Wnt-5a* induces clustering of the scaffold protein PSD-95 in mature hippocampal neurons.** *A*, representative images of neurite immunofluorescence of PSD-95 (*green*) and fluorescence labeling of processes by phalloidin (*blue*) from control medium (*a*), *Wnt-5a* (*b*), or *Wnt-5a* plus sFRP (*c*) after 1 h of treatment. *d*, quantification of the number of PSD-95 clusters/100 μm of neurite ($n = 5$). *B*, time course of the effect of *Wnt-5a* on PSD-95 analyzed by quantification of PSD-95 clusters/100 μm of neurite ($n = 3$). *C*, Western blot of total PSD-95 levels of hippocampal neurons exposed to *Wnt-5a* ($n = 3$). *D*, representative images of dendritic spines of enhanced GFP-expressing neurons (*green*) (*b* and *e*), stained against PSD-95 (*red*) (*a* and *d*), and dendritic spines of enhanced GFP-expressing neurons stained against PSD-95 (*c* and *f*) of hippocampal neurons exposed to *Wnt-5a* (*d–f*) or control conditioned medium (*a–c*) for 2 h. *g*, quantification of PSD-95-positive dendritic spines/10 μm. *Scale bar*, 5 μm. *, $p < 0.01$.

clusters was evidenced. A 25% increase after 30 min was observed, reaching plateau values of 50% over control after 120 min of *Wnt-5a* exposure (Fig. 1*B*). To evaluate whether the increase in the PSD-95 cluster density is due to an increase in the expression of total PSD-95 protein, we evaluated the levels of PSD-95 protein in a total extract of hippocampal neurons. *Wnt-5a* did not change the total levels of PSD-95 in the same temporal course in which we observed the increase in the number of PSD-95 clusters (Fig. 1*C*).

The presence of PSD-95 clusters in excitatory neurons is well correlated with the number of mature dendritic spines (6, 31). To confirm that *Wnt-5a* induces clustering of PSD-95 in dendritic spines, neurons were transfected with the enhanced GFP to analyze the PSD-95 contained in spines. Dendritic protrusions below 3 μm in length showing visible spine heads were evaluated for the presence of PSD-95 immunoreactivity and quantified. Hippocampal neurons exposed to *Wnt-5a* for 2 h presented an 80% increase in the number of PSD-95-containing spines (Fig. 1*D*, *a–g*). These results indicate that *Wnt-5a* but not *Wnt-7a* affects the postsynaptic region of mammalian synapse by promoting the clustering of PSD-95.

New PSD-95 Clusters Induced by Wnt-5a Come from a Diffuse PSD-95 Pool—Since an increase in the clustering of PSD-95 was observed but the total amount of this protein did not change, we asked whether *Wnt-5a* increases the number of PSD-95 clusters due to a redistribution of existing PSD-95. To evaluate changes in the PSD-95 distribution, two hypotheses were considered. First, the new clusters of PSD-95 induced after *Wnt-5a* ligand are due to the splitting of existing PSD-95 clusters (Fig. 2*A*). Second, *Wnt-5a* induces the clustering of PSD-95 through recruitment of free PSD-95 from a diffuse pool (Fig. 2*B*). Hippocampal neurons were incubated with *Wnt-5a*, and different parameters of PSD-95 clusters were analyzed. The mean cluster intensity was evaluated to determine whether *Wnt-5a* decreases the intensity of PSD-95 clusters. Results show that *Wnt-5a* did not affect the mean intensity of PSD-95 clusters (Fig. 2*C*), indicating that the increase in the number of PSD-95 clusters is not due to a decrease in the amount of PSD-95 contained in preexistent clusters. Then in order to analyze whether *Wnt-5a* induces the splitting of existing PSD-95 clusters, the mean PSD-95 cluster area was measured. Moreover, we also quantified the relative PSD-95 cluster integrated intensity. If *Wnt-5a* decreases the mean PSD-95 cluster area and the relative PSD-95 cluster integrated intensity, it is possible that splitting of PSD-95 clusters occurs. However, if the integrated intensity of PSD-95 increases, it will suggest that new PSD-95 clusters come from a diffuse pool. Results show that *Wnt-5a* did not affect the mean area of PSD-95 (Fig. 2*D*) but increased the inte-

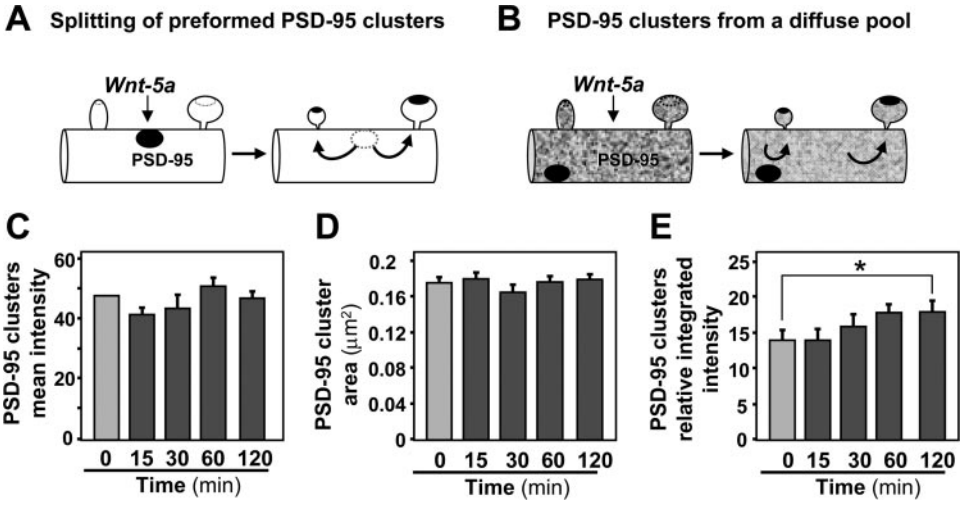

FIGURE 2. ***Wnt-5a* does not use preexistent PSD-95 clusters to form new clusters of PSD-95.** *A* and *B*, models summarizing the two hypotheses raised. *A*, new clusters of PSD-95 induced by *Wnt-5a* are formed by splitting of preexistent clusters. *B*, the clusters of PSD-95 are recruited from the PSD-95 diffuse pool, and the preexistent clusters are not affected. *C–E*, hippocampal neurons exposed to *Wnt-5a* in a temporal course were stained for PSD-95. *C*, mean PSD-95 cluster intensity ($n = 3$); *D*, PSD-95 cluster area ($n = 3$); *E*, relative PSD-95 cluster intensity ($n = 3$). *, $p < 0.05$.

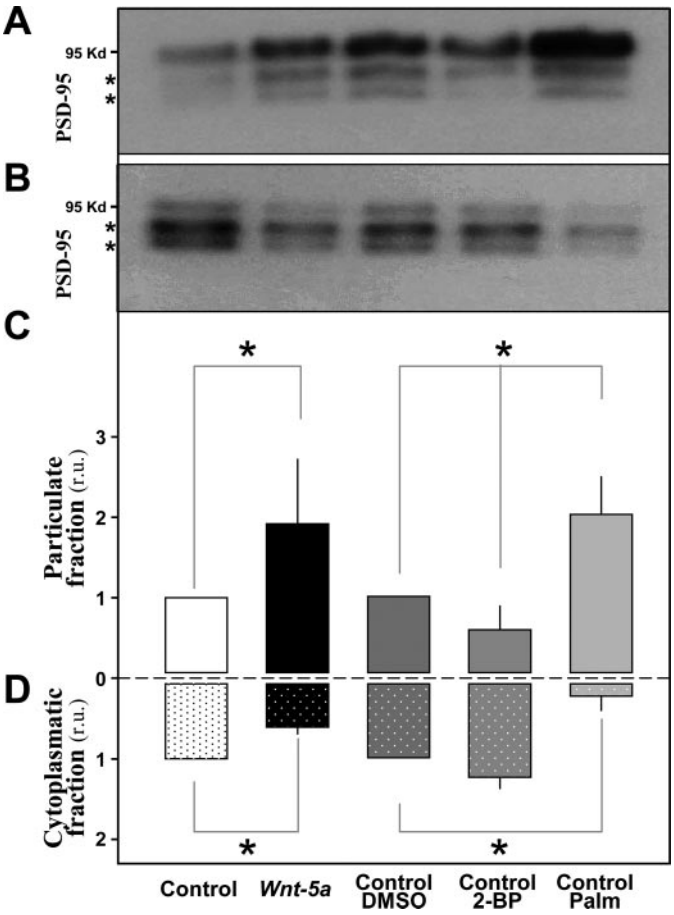

FIGURE 3. **A diffuse pool of PSD-95 is recruited to the membrane pool by *Wnt-5a* ligand.** Hippocampal neurons were incubated in the presence or absence of *Wnt-5a* for 2 h, and the distribution of PSD-95 in the particulate fraction (*A*) and cytoplasmic fraction (*B*) was evaluated by Western blot. As control, hippocampal neurons were incubated with DMSO (vehicle), 2-bromopalmitate (*2BP*), and palmitate (*Palm*) for 4 h, and a subcellular fractionation was performed. *, the cross-reactivity of monoclonal PSD-95 antibody with bands of slightly slower migration that correspond to other MAGUK proteins (46). The mobility of the protein PSD-95 to the particulate fraction under the activity of the *Wnt-5a* ligand was quantified (*C*). The movement of the protein PSD-95 to the particulate fraction was controlled by treatment with 2-bromopalmitate and palmitate. Protein PSD-95 remains in the cytosolic fraction under the activity of the ligand is shown in *D*, also using the palmitate and 2-bromopalmitate controls. Western blots were performed against the protein PSD-95, and the 95 kDa band was quantified ($n = 3$). *, $p < 0.05$.

grated intensity of PSD-95 (Fig. 2*E*), indicating that splitting of preexistent PSD-95 clusters did not occur. These results suggest that new clusters of PSD-95 come from a diffuse dendritic cytoplasmic pool without affecting the size or amount of PSD-95 present in preexistent clusters.

To confirm the hypothesis that *Wnt-5a* induces new PSD-95 clusters from soluble pools of PSD-95 protein (non-membrane attached pool), a subcellular fractionation study was performed.

Previous studies indicated that PSD-95 is a palmitoylated protein that requires palmitate in order to anchor it to the postsynaptic membrane (33). As a control of PSD-95 redistribution between membrane and cytoplasmic pools, hippocampal neurons were incubated with 2-bromopalmitate or palmitate, both substances that affect the interaction of PSD-95 with the membrane. 2-bromopalmitate induces the mobilization of PSD-95 from membrane (Fig. 3, *A* and *C*) to the cytoplasm (Fig. 3, *B* and *D*) and palmitate redistributes PSD-95 from cytoplasm (Fig. 3, *B* and *D*) to the membrane-enriched compartment (Fig. 3, *A* and *C*), as evidenced previously (33, 34). Therefore, we evaluate whether *Wnt-5a* induces a change in the localization of PSD-95. We found that hippocampal neurons treated with *Wnt-5a* for 2 h showed increased levels of PSD-95 in the membrane-enriched fraction with respect to the control (Fig. 3, *A* and *C*) (as assessed by the presence of N-cadherin and transferrin receptor and the absence of GAPDH proteins; data not shown) and a concomitant decrease in the PSD-95 levels in the cytoplasmic fraction (Fig. 3, *B* and *D*) (as assessed by the presence of β-tubulin and GAPDH and the absence of N-cadherin and transferrin receptor proteins; data not shown). These results are consistent with the idea that new clusters of PSD-95 come from a diffuse dendritic cytoplasmic pool.

Wnt-5a Is a Non-canonical Wnt Ligand in Rat Hippocampal Neurons—Different *Wnt* ligands act through canonical or non-canonical *Wnt* pathways, depending on the cellular context (35). To evaluate whether the effect of *Wnt-5a versus Wnt-7a* in the clustering of PSD-95 is due to the activation of different *Wnt* pathways, we incubated mature hippocampal neuron with the *Wnt-5a* and *Wnt-7a* ligands and analyzed the main effectors of the *Wnt* pathways. Previously, we showed that *Wnt-7a* induces the stabilization of β-catenin in a time-dependent manner, where the accumulation of β-catenin became evident after 30 min (23, 27).

Hippocampal neurons exposed to *Wnt-5a* did not show any change in the stabilization of β-catenin even after 2 h of treatment (Fig. 4*A*). Moreover, when *Wnt-5a* and *Wnt-7a* ligands were compared in their capacity to activate the canonical *Wnt* pathway, *Wnt-7a* but not *Wnt-5a* stabilized β-catenin (Fig. 4, *A* and *B*). The co-incubation of the *Wnt-7a* ligand with sFRP prevented the increase in the β-catenin levels at 2 h of treatment (Fig. 4*B*). Since *Wnt-5a* but not *Wnt-7a* induces the clustering of PSD-95, these results suggest that *Wnt-5a* ligand does not require the activation of the canonical *Wnt* pathway to promote the clustering of PSD-95.

Wnt ligands can act through the non-canonical *Wnt* pathways, such as *Wnt*/Ca^{2+} and *Wnt*/JNK (12, 26). To assess whether *Wnt-5a* activates non-canonical *Wnt* pathways in hippocampal neurons, we evaluated CAMKII activation, a downstream component on the Wnt/Ca^{2+} pathway and JNK activation, a downstream component on the *Wnt*/JNK pathway (26).

Hippocampal neurons incubated with *Wnt-5a* showed phosphorylation of CAMKII after 5 min, with a peak at 15 min, and then phosphorylation decreased at 30 min and remained at similar levels at 1 h with respect to control conditions (Fig. 4*C*). When the phosphorylation state of JNK was evaluated, we observed that *Wnt-5a* increased the phosphorylation of JNK (46-kDa isoform) after 30 min with a peak at 60 h (Fig. 4*D*), similar to the observed increase in the PSD-95 clustering. The total levels of JNK were not affected by *Wnt-5a* treatment (Fig. S2). The effect on JNK phosphorylation was antagonized by co-incubation with the *Wnt* antagonist sFRP at 2 h of treatment (Fig. S3).

Since we have previously not found changes in the clustering of PSD-95 in the presence of *Wnt-7a* ligand, we examined whether *Wnt-7a* ligand can activate the *Wnt*/JNK pathway.

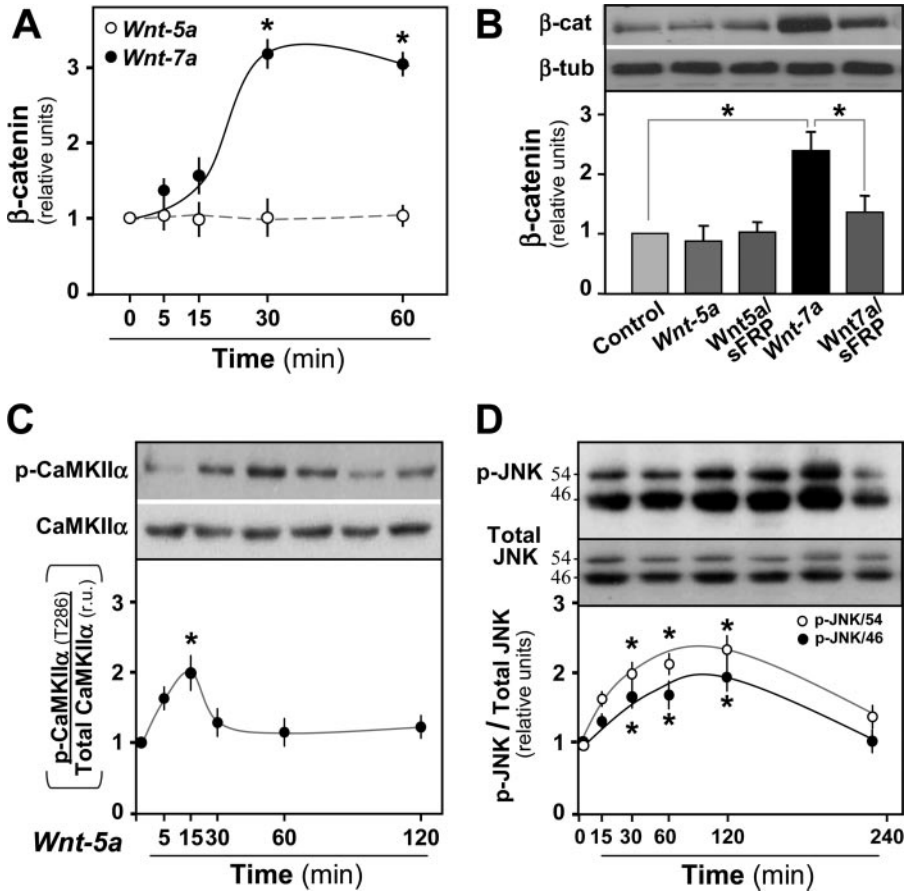

FIGURE 4. ***Wnt-5a* activates noncanonical *Wnt* pathways on 14-DIV hippocampal neuron cultures.** *A*, quantification of Western blot of β-catenin in hippocampal neurons exposed to *Wnt-5a* and *Wnt-7a* in a temporal course ($n = 3$). *B*, representative Western blot of β-catenin in neurons stimulated with *Wnt-5a*, *Wnt-7a*, or both, co-incubated with sFRP for 1 h ($n = 4$). *C*, representative Western blot of total and phosphorylated CAMKII in neurons treated for different times with *Wnt-5a* ligand ($n = 3$). *D*, representative Western blot of total and phosphorylated JNK in neurons stimulated with *Wnt-5a* for different times. In the *graphs*, JNK2/3 = 55 kDa and JNK1 = 46 kDa. ($n = 5$). *, $p < 0.05$.

Interestingly, we have not observed phosphorylation/activation of JNK by *Wnt-7a* at 1 h of treatment (data not shown) (23), suggesting that *Wnt-5a* signaling through a noncanonical *Wnt* pathway promotes the clustering of PSD-95 in hippocampal neurons.

The Non-canonical Wnt/JNK Pathway Is Involved in the PSD-95 Clustering Induced by Wnt-5a—To determine the contribution of the non-canonical *Wnt* pathways in the clustering of PSD-95 induced by *Wnt-5a*, we used fumagillin, a synthetic inhibitor of the non-canonical *Wnt* pathways that acts downstream of the *Wnt* receptors but upstream of CAMKII and JNK without affecting the canonical *Wnt*/β-catenin pathway (36) (Fig. 5*A*). In hippocampal neurons, fumagillin blocked the activation of JNK induced by *Wnt-5a* and did not affect the β-catenin stabilization (Fig. 5*B*). Interestingly, fumagillin inhibited the clustering of PSD-95 induced by *Wnt-5a* at 1 h, without affecting the clustering of PSD-95 (Fig. 5*C*) or the dendritic spine formation (Fig. S4) with fumagillin alone. To dissect whether *Wnt-5a* induces the clustering of postsynaptic proteins via *Wnt*/Ca^{2+} or *Wnt*/JNK pathways, we have used inhibitors for the three well known effectors of these pathways: KN-93 and BSD-X to inhibit the activity of CAMKII and protein kinase C, respectively, of the *Wnt*/Ca^{2+} pathway and SP600125 to inhibit the activity of JNK of the *Wnt*/JNK pathway (Fig. 5*A*).

Hippocampal neurons were incubated with *Wnt-5a* ligand for 1 h in the presence of KN-93 or BSD-X. However, the *Wnt*/Ca^{2+} pathway inhibitors did not affect significantly the PSD-95 clustering induced by *Wnt-5a* (Fig. 6, *A* and *B*). However, when neurons were co-incubated with *Wnt-5a* in the presence of SP600125, the clustering of PSD-95 induced by *Wnt-5a* was completely blocked (Fig. 7, *A* and *B*). To confirm that the *Wnt*/JNK pathway is implicated in the clustering of PSD-95, we used anisomycin, a JNK activator (37, 38). Anisomycin induced the clustering of PSD-95 at 1 h of treatment in a way similar to that of *Wnt-5a* ligand (Fig. 7, *A* and *B*).

Since pharmacological reagents as SP600125 and anisomycin may cause nonspecific effects, we used a small peptide that inhibits JNK activity, and it is structurally unrelated to SP600125. TAT-TI-JIP is a smaller 11-mer peptide of JIP conjugated to Tat peptide that perturbs the interaction of JIP-JNK and, as a consequence, affects the JNK activity (39). We observed that TAT-TI-JIP prevented the increase in the number of PSD-95 clusters induced by *Wnt-5a* in a concentration-dependent manner (Fig. 7, *C* and *D*) under conditions in which TAT-TI-JIP alone at this concentrations did not affect the clustering of PSD-95 (Fig. 7, *C* and *D*). These results indicate that the non-canonical *Wnt*/JNK pathway is required for the clustering of the PSD-95 induced by the *Wnt-5a* ligand.

Wnt-5a Increases Synaptic Amplitude of the Glutamatergic Transmission without Affecting Paired Pulse Facilitation—To examine the effects of *Wnt-5a* on excitatory glutamatergic transmission evoked by stimulation of Schaeffer collaterals in adult hippocampal slices, we recorded the fEPSP in presence of picrotoxin (10 μM) to block $GABA_A$-mediated inhibitory synaptic transmission. The fEPSP amplitude increased within 20 min after the addition of *Wnt-5a* conditioned medium to ACSF perfusion medium, without changing either the fiber volley (*fV*) amplitude (Fig. 8, *B* and *E*) or PPF (Fig. 8*D*). This effect was antagonized completely in the presence of an anti-*Wnt-5a*, a generic antibody against the *Wnt-5a* domain (Fig. 8, *A* and *C*). On average, *Wnt-5a* induced an increase 205.2% ± 42.4 of the fEPSP amplitude, which was blocked in the presence of anti-*Wnt-5a* (Fig. 8*C*), whereas the mean values of the PPF and the fiber volley amplitude did not change significantly with respect to their controls in any of these conditions (Fig. 8, *B*, *D*, and *E*).

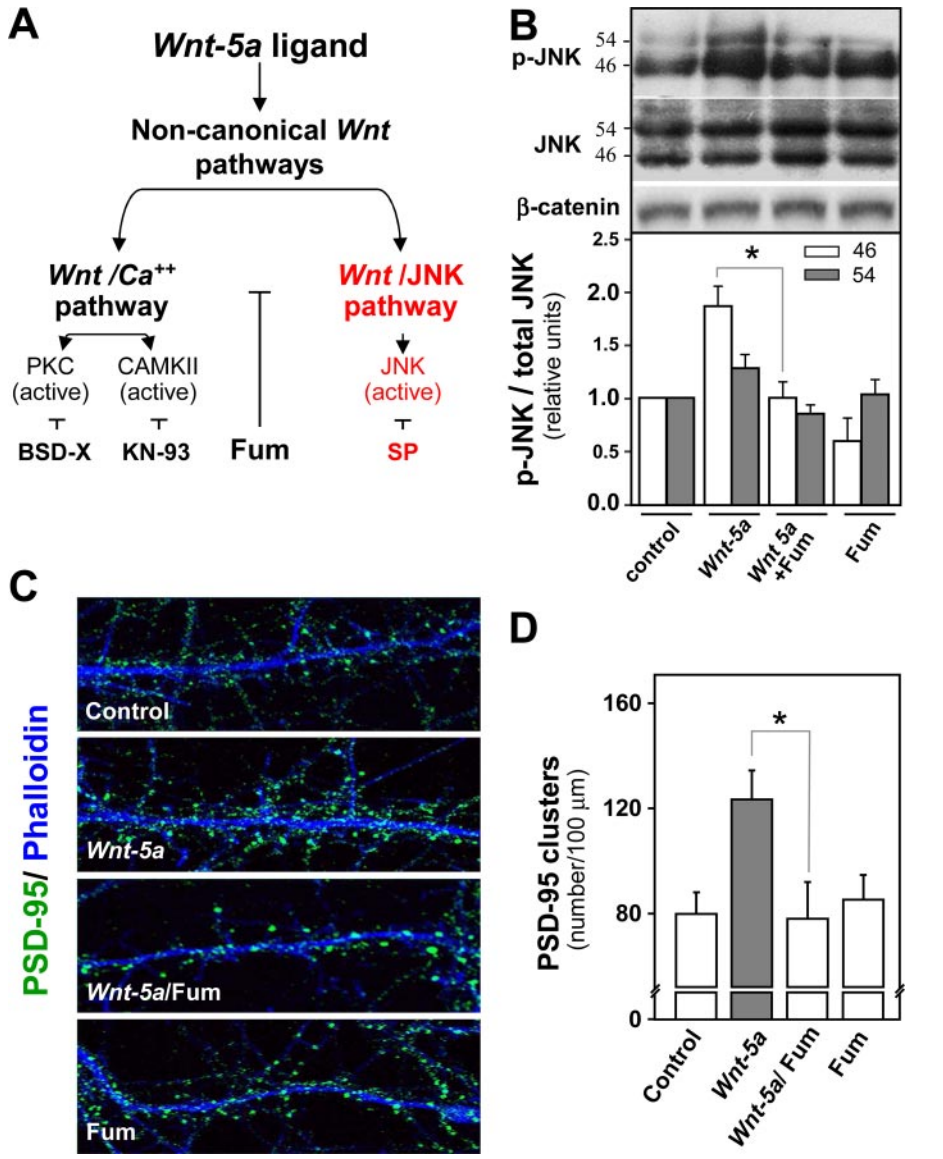

FIGURE 5. **Non-canonical pathway signaling is involved in the clustering of PSD-95 induced by *Wnt-5a*.** *A*, the *scheme* indicates the two non-canonical *Wnt* pathways described for *Wnt-5a*: *Wnt*/Ca^{2+} and *Wnt*/JNK. The effectors for each pathway (CAMKII, protein kinase C, and JNK) are shown as well as the drugs used to inhibit these kinases: KN-93, BSD-X, and SP. Fumagillin (*Fum*) is a general inhibitor of the noncanonical *Wnt* pathways. *B*, representative Western blot of β-catenin, phospho-JNK, and JNK of neurons treated with *Wnt-5a* for 1 h in the presence or absence of fumagillin or control medium with o without fumagillin. The *graph* shows the quantification of phospho-JNK normalized against JNK. *C*, representative neurite images of hippocampal neurons exposed to *Wnt-5a* for 1 h in the presence or absence of fumagillin or control medium with or without fumagillin. *D*, quantification of PSD-95 clusters/100 µm of neurite shown in *C* ($n = 3$). *, $p < 0.05$.

These results indicate that *Wnt-5a* does not affect the presynaptic region to induce a increase in the fEPSP, suggesting that *Wnt-5a* plays a functional role in the postsynaptic region.

DISCUSSION

Maturation of functional postsynaptic compartments involves gradual recruitment of scaffold molecules, receptors, and a specialized signaling machinery for clusters in dendrites, which then will form part of functional synapses or be eliminated (40–42). This process is highly regulated for transynaptic adhesion molecules and secreted factors that ensure the coordination with the presynaptic differentiation status or activity. So far, agrin and narp are the only soluble synaptogenic factors that modulate postsynaptic differentiation in developing central nervous system synapses acting only on the postsynaptic side (4). Previous studies of Budnik and co-workers (43) on the *Drosophila* neuromuscular junction indicated that the loss of *Wingless* results in aberrant development of postsynaptic specializations.

Here, for the first time it is shown that a *Wnt* ligand is able to modulate the postsynaptic region in the mammalian central nervous system. Since the presynaptic and postsynaptic regions strongly interact, alterations in structuring the presynaptic terminal or the postsynaptic region are accompanied by a parallel change in the opposite synaptic side. Our studies were made after short term exposure to *Wnt* ligands; however, we do not know whether a long term exposure to *Wnt* ligands affects the presynaptic counterpart as a consequence of postsynaptic differentiation.

Ataman *et al.* (44) found that *Wg* (*Wingless*) acts through different pathways to regulate pre- and postsynaptic boutons in glutamatergic *Drosophila* neuromuscular junction. However, in mammals, different *Wnt* ligands are expressed. Interestingly, our group have found that different *Wnt* ligands operate in the central nervous system synapse. *Wnt-7a* and *Wnt-3a*, but not *Wnt-5a*, activate the canonical *Wnt* pathway and regulate the presynaptic region modulating the clustering of presynaptic proteins, including α7-nAChR (27) and the synaptic vesicle cycle in hippocampal neurons (23). Here, we showed that *Wnt-5a* but not *Wnt-7a* regulates the postsynaptic region promoting the clustering of PSD-95 protein through activation of the non-canonical *Wnt* pathway. However, other *Wnt* ligand could be involved in the modulation of the postsynaptic region through activation of non-canonical *Wnt* pathways.

The increase in the number of PSD-95 clusters induced by *Wnt-5a* observed in mature hippocampal neurons suggests an increase in the number of dendritic spines. We found that almost 80% of dendritic spines contain PSD-95 clusters under control conditions (data not shown) and that *Wnt-5a* induces the number of total protrusions (data not shown) and protrusions containing PSD-95 by ~60%. It is possible that the increase in the number of clusters of PSD-95 will be contained in newly formed protrusions;

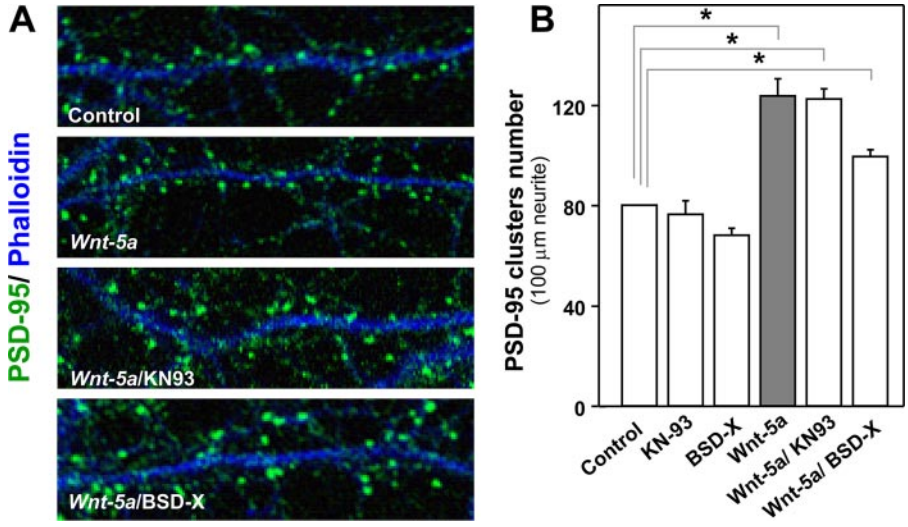

FIGURE 6. **Inhibition of two effectors of the non-canonical *Wnt*/Ca^{2+} pathway (CAMKII and protein kinase C) does not affect the clustering of PSD-95 induced by *Wnt-5a*.** *A*, representative neurite images of hippocampal neurons exposed to *Wnt-5a* for 1 h in the presence or absence of KN-93 or BSD-X or control medium with or without KN93 or BSD-X are shown. *B*, quantification of the immunofluorescence of PSD-95 clusters/100 μm of neurite of the treatment indicated in *A* ($n = 3$). *, $p < 0.05$.

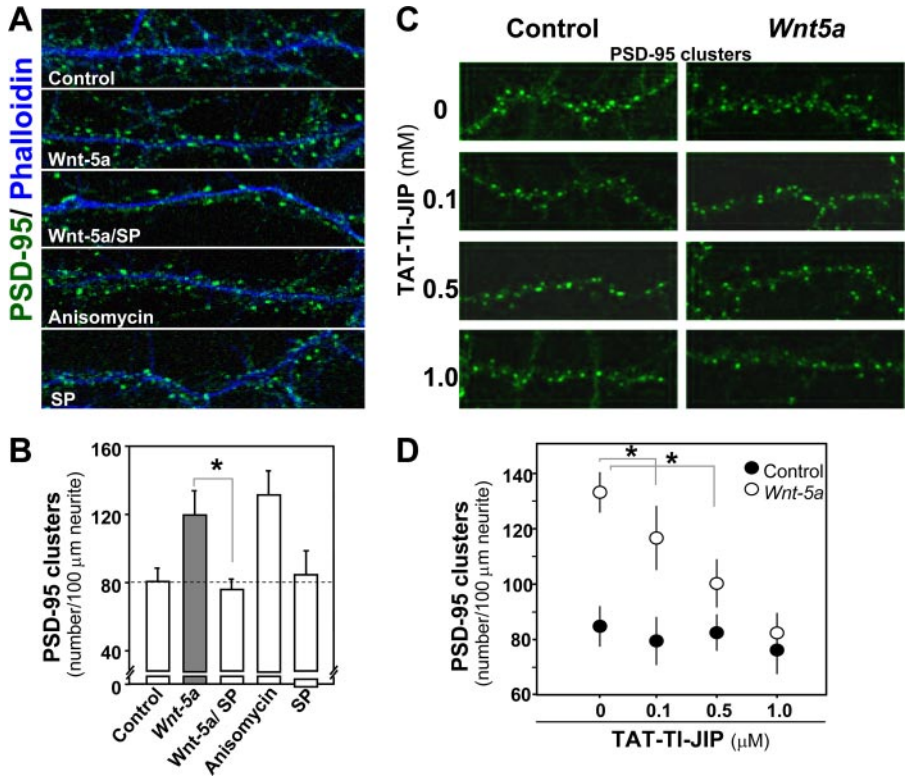

FIGURE 7. ***Wnt-5a* induces the clustering of PSD-95 through activation of the *Wnt*/JNK non-canonical pathway.** *A*, representative neurite images of hippocampal neurons treated with or without *Wnt-5a* for 1 h co-incubated or not with SP600125 (*SP*). Neurons were treated with anisomycin for 1 h as a control for JNK activation. *B*, quantitative immunofluorescence of PSD-95 clusters/100 μm of neurite exposed to the different treatments ($n = 3$). *C*, representative neurite images of hippocampal neurons treated with different concentrations of the TAT-TI-JIP peptide in the presence of *Wnt-5a* or pcDNA. *D*, quantitative immunofluorescence of PSD-95 clusters number/100 μm of neurite exposed to treatments indicated in *C* ($n = 3$). *, $p < 0.05$.

however, it is necessary to evaluate the kinetics of the event to know for sure whether the PSD-95 is recruited as a cluster concomitantly or after the protrusion formation.

Interestingly, we found that *Wnt-5a* can increase the number of PSD-95 clusters without changing the total expression of PSD-95 and that *Wnt-5a* increases the density of PSD-95 clusters due to a redistribution of existing PSD-95. Two ideas for *de novo* cluster formation of PSD-95 have been suggested. It has been observed that splitting of PSD-95 clusters can occur in neuron cultures (45). On the other hand, *de novo* PSD-95 clusters can be formed by recruitment from a diffuse pool of PSD-95 into the synapse with no obvious involvement of discernible discrete transport particles (46).

Our quantitative analysis of PSD-95 clusters suggests that splitting of PSD-95 cannot explain the increase in PSD-95 clustering induced by *Wnt-5a*, because the relative cluster integrated intensity of PSD-95 was increased and the mean size of the PSD-95 cluster did not change. These results are consistent with the idea that new PSD-95 clusters induced by *Wnt-5a* could be formed from a diffuse pool of PSD-95. Subcellular fractionation corroborates our results. In fact, we observed an increase in the membrane-attached PSD-95 pool (particulate fraction) and a decrease in the diffuse PSD-95 pool (cytoplasmic fraction). The monoclonal antibody to PSD-95 cross-reacted with bands of slower migration, which have been described as part of the MAGUK family (47). These proteins also are affected by the *Wnt-5a* ligand, suggesting that PSD-95-related members are modulated by *Wnt-5a*; however, more studies are required to clarify this issue.

In this study, we showed that SP600125, a specific JNK inhibitor (48), and TAT-TI-JIP, a small peptide that perturbs the interaction of JIP-JNK affecting the JNK activity (39), prevent the increase in the clustering of PSD-95 induced by *Wnt-5a*, suggesting that a *Wnt*/JNK signaling pathway is required by *Wnt-5a* to induce the clustering of PSD-95 in hippocampal neurons. Moreover, we have demonstrated that the treatment of hippocampal neurons with a JNK activator (anisomycin) and a mimetic formylated hexapeptide can imitate both the effect of *Wnt-5a* on PSD-95 clustering. A higher concentration of SP (5–20 μM) as used by Rosso *et al.* (37) and Kim *et al.* (38) or

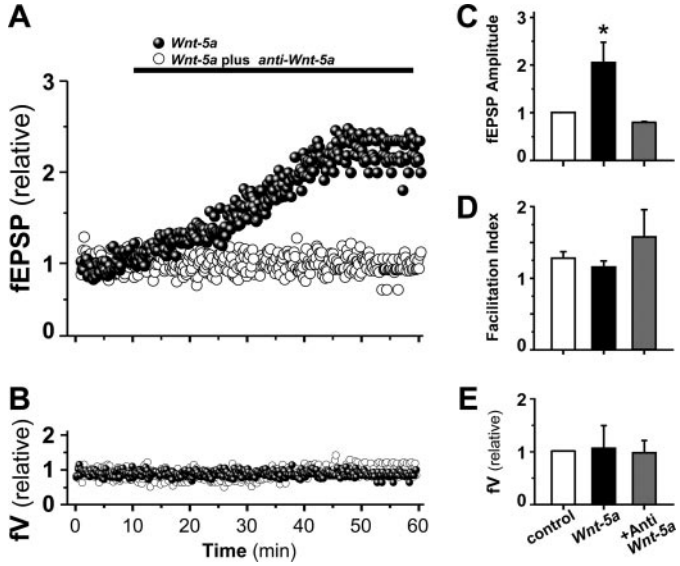

FIGURE 8. **Field Potential recording of a CA1 pyramidal cell after *Wnt-5a* treatment.** *A*, field recording of CA1 pyramidal cells treated with *Wnt-5a* or *Wnt-5a* plus anti-*Wnt-5a* antibody. Time course of fEPSP effect of *Wnt-5a* (*black spheres*) or *Wnt-5a* plus anti-*Wnt-5a*. *B*, effect of *Wnt-5a* on fiber volley. *C*, normalized amplitude of fEPSPs, evoked by the first stimulus, in control and after *Wnt-5a* or *Wnt-5a* plus anti-*Wnt-5a* treatment after 50 min of continued perfusion ($n = 3$). *D*, index of facilitation, measured before (control) and after *Wnt-5a* or *Wnt-5a* plus anti-*Wnt-5a* treatment application ($n = 3$). *E*, average values of normalized amplitude of fiber volley in both conditions are shown ($n = 3$). *, $p < 0.05$.

TAT-TI-JIP (5 μM), affected the clustering of PSD-95 (data not shown), according with an endogenous JNK activity that maintains the postsynaptic structure in hippocampal neurons.

The increase in the clustering of PSD-95 induced by the *Wnt-5a* ligand is correlated with the increase in mature dendritic spines with PSD-95 positive in their heads, suggesting that the non-canonical *Wnt*/JNK signaling participates in the maturation of the overall postsynaptic structure. Previous evidence suggests that PSD-95 palmitoylation is required to move PSD-95 from a diffuse pool to the synaptic membrane (33). A recent study also suggests that phosphorylation of Ser-295 of PSD-95 is necessary to anchor PSD-95 to the postsynaptic membrane (38). We have also demonstrated that *Wnt-5a* activates preferentially the p46 isoform of JNK. This is very interesting, because the phosphorylation of serine 295 in PSD-95 by JNK-1 (p46) has been suggested to regulate the synaptic accumulation of PSD-95 (38). Moreover, Rosso *et al.* (37) indicated that *Wnt-7b* mediates dendritic development in hippocampal neurons by activating a JNK signaling that will be involved in the regulation of the neuronal cytoskeleton. We do not discard the possible contribution of the JNK-mediated regulation of cytoskeleton to the effects of *Wnt-5a* on the PSD-95 clustering. The mechanism used by the *Wnt-5a*/JNK pathway to form clusters of PSD-95 remains unknown; however, it is highly possible that one of these targets could allow the movement of PSD-95 from a diffuse pool in the cytoplasm to a membrane environment.

PSD-95 regulates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA receptor) insertion and dendritic spine morphology changes that occur during synaptic plasticity. It has been demonstrated that overexpression of PSD-95 converts silent to functional synapses and increases the amplitude and frequency of mEPSP (49). Moreover, inhibition of PSD-95 palmitoylation decreases AMPA receptor-mediated miniature postsynaptic currents (33). Several publications show that the activation of the *Wnt* pathway has relevance in the synaptic process. *Wnt-3a* has a regulatory role in long term potentiation (17), an electrophysiological correlate of memory encoding, suggesting a role for *Wnt* signaling in the regulation of synaptic plasticity (5). Small synthetic molecules mimic *Wnt*, leading to both increased spontaneous and evoked neurotransmission that occurs in a transcription-independent fashion (51). Functionally, our results indicate that *Wnt-5a* is able to increase the amplitude of fEPSP after 20 min of treatment of hippocampal slices with no effect on presynaptic activity, suggesting that *Wnt-5a* plays a functional role in the postsynaptic region, probably as a consequence of the increase on PSD-95 clustering.

In the canonical *Wnt* pathway, it has been reported that members of the LRP family of single pass transmembrane proteins are also required for the reception of the *Wnt* signal transduction pathway and that both Fz and LRP function as co-receptors for *Wnt* proteins (52). A recent study indicates that *Wnt-5a* either activates or inhibits β-catenin-TCF signaling, depending on the receptor context (35), suggesting that a *Wnt* ligand may act in a canonical or non-canonical form, depending on the corresponding characteristics of the receptor present in the cell. In hippocampal neurons, the *Wnt-5a* ligand and its formylated mimetic were able to modulate non-canonical *Wnt* pathway components but did not affect canonical *Wnt* pathway molecules, such as β-catenin. Therefore, it is possible that the LRP receptor may not be present in a context that allows *Wnt-5a* to act as a canonical *Wnt* ligand in hippocampal neurons.

Interestingly, it has been reported that the canonical *Wnt* pathway is required specifically in the presynaptic region to promote synaptic differentiation at the *Drosophila* neuromuscular junction and that a loss of function of this pathway in the postsynaptic region does not affect this process (53). In our laboratory, we showed that *Wnt-7a* ligand increases the frequency of fEPSP and field excitatory postsynaptic currents through presynaptic mechanisms (23), and our experiments showed that *Wnt-5a* increases the amplitude of the fEPSP, suggesting that the mechanism of *Wnt-5a* is postsynaptic because the PPF is not affected. This evidence together with our results suggest that the canonical and non-canonical *Wnt* pathways are compartmentalized to act locally and coordinate the differentiation of the pre- and postsynaptic region, respectively.

The fact that *Wnt-5a* and their formylated hexapeptide-derived ligands promote the clustering of PSD-95 suggests that the non-canonical *Wnt* signaling pathway may be a target for the treatment of neurodegenerative diseases that affect synaptic integrity, such as Alzheimer disease (32, 50, 54).

Acknowledgments—We thank Dr. P. C. Salinas, R. Moon, and J. Nathans for providing the Wnt-7a, Wnt-5a, and sFRP-1 HA constructs.

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